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Short Communication

Cotinine determination by immunoassays may be influenced by other nicotine metabolites

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Abstract. Polyclonal rabbit anticotinine antiserum, which can be used for biomonitoring nicotine uptake by the determination of cotinine in body fluids, was checked by a competitive ELISA for its cross-reactivity with nine nicotine metabolites. The highest percentage of relative cross-reactivity (about 30%) was observed with trans-3'-hydroxycotinine, a metabolite which is known to be excreted in 3-fold higher amounts than cotinine in the urine of human smokers. Therefore, it is possible that cotinine determinations performed by immunochemical methods – especially in urine – may yield overestimated cotinine concentrations.

Key words: Cotinine – 3'-Hydroxycotinine – Cross reaction – Enzyme-linked immunosorbent assay – Biomonitoring

Introduction

Several parameters have been proposed for biomonitoring the exposure of individuals to tobacco smoke. They have been reviewed recently by the International Agency for Research on Cancer (IARC monographs 1986), and with respect to environmental tobacco smoke by the National Research Council (NRC 1986) and Jarvis (1987). Among those parameters, the determination of nicotine uptake is the one most recommended, since nicotine is rather specific for tobacco smoke. Nicotine itself has a short biological half-life (about 1–2 h in human plasma; Benowitz et al. 1982; Kyerematen et al. 1982, Luck and Nau 1984). Since cotinine, a nicotine metabolite, has the advantage of a longer plasma half-life of 16–20 h (Benowitz et al. 1983), it has been widely used as a marker for nicotine uptake.

Sensitive methods for the determination of cotinine in biological fluids are available. Some of these are based on immunochemical techniques using specific antibodies. The antibodies used, however, should not cross-react with other compounds possibly present in the test fluid. Therefore, the mono- or polyclonal anticotinine antibodies have been tested for cross-reactivity with nicotine and nicotine metabolites (Langone et al. 1973; Langone and van Vunakis 1982; Bjerke et al. 1986, 1987; Hansel et al. 1986; van Vunakis et al. 1987).

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However, in none of these studies were the used anticotinine antisera tested for cross-reactivity with trans-3'-hydroxycotinine (THOC), a nicotine metabolite which is excreted in amounts 3 times higher than cotinine in the urine of human smokers (Neurath et al. 1987). The objective of this study was to investigate in a competitive cotinine ELISA the cross-reactivity of an anticotinine antiserum which was raised in rabbits according to the method described by Langone et al. (1973). Special emphasis was placed on cross-reactivity with the nicotine metabolite THOC.

Material and methods

Materials. The anticotinine antiserum was raised in rabbits by immunization with a thyroglobulin-cotinine conjugate. The conjugates of cotinine with thyroglobulin or human serum albumin (HSA) were prepared by reaction with trans-4'-carboxycotinine as described by Langone et al. (1973). Alkaline phosphatase-conjugated antirabbit IgG (goat) was purchased from Medac (Hamburg, FRG). *p*-Nitrophenylphosphate and the diethanolamine buffer were supplied by Boehringer (Mannheim, FRG), bovine serum albumin (BSA, fraction V) by Baker (Gross-Gerau, FRG), HSA by Sigma (Deisenhofen, FRG), and thyroglobulin and (−)-nicotine by Serva (Heidelberg, FRG). (−)-Cotinine was purchased from Roth (Karlsruhe, FRG). (−)-nicotine-1'-N-oxide, trans-4'-carboxycotinine, (±)-cis- and (±)-trans-3'-hydroxycotinine, (±)-demethylcotinine, and (−)-cotinine-1-N-oxide from Dr Georg Neurath (Hamburg, FRG). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

Buffers. PBS: phosphate buffered saline, 0.002 mol/l phosphate, 0.15 mol/l NaCl, pH 7.4; PBS-T: PBS supplemented with 0.05% (v/v) Tween 20; PBS-BSA-T: PBS-T supplemented with 0.1% (w/v) BSA.

Methods. The competitive ELISA for cotinine determination was performed in 96-well polystyrene microtiter plates (Immunoplate I, Nunc, Wiesbaden, FRG). The plates were coated with 50 µl cotinine-HSA conjugate diluted in 0.1 mol/l carbonate-bicarbonate buffer, pH 9.6, by incubation over night at 4° C. The wells were washed three times with PBS-T. Competitor solutions (100 µl) of various concentrations (concentration range see Table 1) were preincubated with 150 µl anticotinine antiserum

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Table 1. Relative cross-reactivity of anticotinine antibodies with nicotine metabolites

Compound	Concentration in assay (mol/l)			Steepness of inhibition curve[a] (absorbance units/log (mol/l))	Relative cross-reactivity (%)		
	Min. ($\times 10^{-9}$)	Max. ($\times 10^{-3}$)	At 50% inhibition ($\times 10^{-6}$)		This study[b]	Published data	
						Langone et al. (1982)[b]	Bjercke et al. (1986)[c]
(−)-cotinine	57	0.057	3.5	−0.40	100	100	100
1-N-methylnicotinium iodide	33	10	–	–	< 0.037	–	–
1'-N-methylnicotinium iodide	33	10	13 100	−0.58	0.027 ± 0.006[d]	–	–
nicotine-1'-N-oxide	56	17	9 660	−0.54	0.053 ± 0.006[d]	0.01	0.025
(−)-cotinine-1-N-oxide	52	16	1 880	−0.55	0.19 ± 0.01[d]	0.1	–
(−)-nicotine	120	10	1 360	−0.55	0.26 ± 0.04[d]	0.05	0.025
(+)-demethylcotinine	62	19	53.5	−0.39	6.0 ± 1.0[d]	0.5	6.9
(−)-cis-3'-hydroxycotinine	52	16	30.7	−0.48	11.5 ± 1.1[d]	–	–
(+)-trans-3'-hydroxycotinine	52	16	11.8	−0.45	29.6 ± 1.1[d]	–	–
(+)-nicotine	–	–	–	–	–	< 0.01	0.034
(+)-cotinine	–	–	–	–	–	< 0.01	–
(−)-nornicotine	–	–	–	–	–	0.01	0.20
(−)-cotinine-N'-oxide	–	–	–	–	–	–	2.8

Only published data regarding those metabolites which have an intact pyridine and pyrrolidine ring structure were selected

[a] At 50% inhibition

[b] Polyclonal antibodies raised in rabbits

[c] Monoclonal antibodies

[d] Mean ± standard deviation, the coefficients of variation range between 3.7% (for trans-3'-hydroxycotinine) and 22.2% (for 1'-N-methylnicotinium iodide)

(1:4000 diluted with PBS-BSA-T) for 30 min at room temperature (RT). After pipetting 50 µl of the preincubation mixtures into the wells of the coated microtiter plates, these were incubated for 1 h at RT and washed three times with PBS-T. Finally, 50 µl alkaline phosphatase conjugated antirabbit IgG (1:2000 diluted with PBS-BSA-T) was added to each well and the microtiter plate was incubated for 1 h at RT. After the plates were washed three times with PBS-T and one additional time with bidistilled water, 50 µl of freshly prepared substrate solution (10 mmol/l in diethanolamine buffer, pH 9.8, containing 0.5 mmol/l magnesium chloride) was added to each well. The enzyme reaction was performed for 30 min at RT in the dark and was stopped by adding 50 µl of 20% (w/v) NaOH solution. Absorbance was read at 405 nm.

For optimal comparison each individual nicotine metabolite was assayed together with cotinine on one microtiter plate. Duplicate or triplicate assays were performed on different days. The inhibition curves for cotinine and the other nicotine metabolites were calculated by a least square fit to the logistic function $y = A/(1+(B/x)^C)$ in which B represents the logarithm of competitor concentration at 50% inhibition. The logarithm of the competitor concentration and the value of the absorbance of the sample are given by x and y. A corresponds to the maximum absorbance (i.e. that at zero competitor concentration) and C determines the curvation of the concentration-response curve. The relative cross-reactivity was calculated from the molar competitor concentrations at 50% inhibition (Abraham 1969).

Results and Discussion

The nicotine metabolites demethylcotinine and cis- and trans-3'-hydroxycotinine showed moderate to marked cross-reactivity to the anticotinine antiserum (Table 1). It is of special interest that the highest cross-reactivity of about 30% was observed with THOC. High cross-reactivity would lead to an overestimation of cotinine in the presence of THOC and thus would explain the variations between the results which were obtained by determining the cotinine concentrations in urine by immunochemical versus gas chromatographic methods (Johnson et al. 1985; Biber et al. 1987; Letzel et al. 1987). Benowitz et al. (1983) has already discussed the possibility that the discrepancy between the cotinine half-life determined by gas chromatography ($t_{1/2}$ = 16–20 h) and that determined by radioimmunoassay ($t_{1/2}$ = 30–40 h, Langone et al. 1973) might be due to the different methods. In addition, they expected that "metabolites cross-reacting with cotinine antibodies might give a false long estimate of $t_{1/2}$ when measured by radioimmunoassay". The monoclonal anticotinine antibodies which have been produced and described (Bjercke et al. 1986) had not been tested for the cross-reactivity with THOC. Furthermore, these monoclonal antibodies do not seem to be more specific than the polyclonals raised in rabbits (Table 1). Additionally, anticotinine antibodies might have a high stereoselective specificity, as has been shown by Langone et al. (1982) for (+)- and (−)-cotinine (Table 1). Such a stereoselective specificity of anticotinine antibodies cannot be excluded for the cross-reactivity with (+)- and (−)-THOC. Since in the present study the cross-reactivity of THOC was assayed with its racemic form (±)-THOC, and since the naturally occurring metabolite (−)THOC (trans-1-methyl-3-(R)-hydroxy-5-(S)-3-pyridyl-2-pyrrolidinone, Dagne and Castagnoli 1972) is only part of the racemic (±)-THOC, the relative cross-reactivity of the anticotinine antiserum with the (−)-THOC enantiomer might be even higher than 30%.

Cross reactivity of anticotinine antibodies with further nicotine metabolites in the biological test fluids cannot be excluded, especially since, e.g. the whole spectrum of m...

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tine metabolites in the urine of smokers is not yet known (Neurath and Pein 1987). Therefore, one should be aware that biomonitoring the nicotine uptake by the determination of cotinine using immunochemical methods may produce questionable results concerning the cotinine concentrations.

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